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Caloric Restriction Increases the Expression of Heat Shock Protein in the Gut

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Objective

The authors determined whether caloric restriction (CR), either acutely or chronically, alters heat shock protein 70 (*hsp*70) gene expression in the gut.

Summary Background Data

Caloric restriction prolongs the life span and delays age-related disease (*e.g.*, cancer) in mammals; the mechanisms responsible for these effects are not known. Heat shock proteins are a group of stress-responsive genes of which the most prominent member is *hsp*70.

Methods

In the first experiment, adult (4-month-old) rats (n = 3/group) were killed after a 48-hour fast or 6 and 24 hours after refeeding. In addition, three rats (controls) were killed without fasting or refeeding. The stomach was removed and RNA was extracted for *hsp*70 gene expression. In the second experiment, aged (22- to 26-month-old) rats were fed *ad libitum* (AL) or a CR diet (60% caloric intake of AL diet). Rats were killed, the stomach and duodenum were removed, and RNA was extracted for determination of *hsp*70 gene expression.

Results

In the first experiment, *hsp*70 mRNA levels were increased approximately threefold in the stomach of rats fasted for 48 hours; levels decreased to control values by 6 and 24 hours after refeeding. In the second experiment, *hsp*70 mRNA levels were increased significantly in both the stomach and duodenum of aged CR rats compared with AL controls.

Conclusions

The authors have demonstrated that *hsp*70 mRNA levels are increased in the proximal gut of young and old rats, either acutely (with fasting) or with CR. Increased expression of the cytoprotective *hsp*70 gene in the gut may provide a possible cellular mechanism for the beneficial effects noted with CR.

Lifelong caloric restriction (CR) (to approximately 60% of *ad libitum* [AL] intake) is the only known experimental manipulation that can extend the mean and maximum life span and decrease the onset of age-related diseases such as cardiomyopathy, arteriosclerosis, and cancer in experimental animals.[1-3] The mechanisms by which CR retards senescence (and the pathogenesis of aging itself) are not known. Caloric restriction lowers the body set point for basal temperature and shifts nutrient utilization from carbohydrate to fat metabolism, thus providing possible mechanisms for the beneficial effects noted with CR.[4,5] Another current theory, often called the free radical theory of aging,[6,7] contends that free radical reactions underlie the aging process; CR may retard or prevent the age-related decreases in the cellular factors that function as cytoprotective agents in the body. Identifying these specific mechanisms will provide a better understanding of the aging process and possible ways of delaying this physiologic process.

Various studies suggest that the expression of heat shock proteins (Hsps) represents an essential mechanism by which cells cope with stress.[8,9] Discovered originally as intracellular proteins that increase after exposure to heat and act to confer cellular protection against hyperthermia, the Hsps are induced by a variety of cellular stressors such as heavy metals, amino acid analogues, and free radicals, thus, the Hsps also are known as stress-induced proteins.[10-13] Several (approximately 50) Hsps have been identified and are classified into families according to their sizes.[10,11] The most prominent and the most evolutionary-conserved Hsp in humans and other mammals is Hsp70. Hsp70 binds and protects nascent peptides, aids in the translocation of proteins between cellular organelles, and participates in the refolding of proteins after denaturation.[14,15] Recent studies have demonstrated an increase in *hsp*70 expression in the hypothalamus and livers of rats maintained on chronic CR[3,16,17]; however, the effects of CR on *hsp* gene expression in the gut have not been studied.

The purpose of this study was twofold. We sought to determine: 1) whether fasting and refeeding altered *hsp* gene expression in the gut of young adult (4-month-old) rats, and 2) whether short-term CR (60% of *ad libitum*-fed animals) alters *hsp*70 mRNA levels in the gut. Increases in *hsp* levels could confer a cytoprotective effect in the gut of rats treated by CR.

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MATERIAL AND METHODS

Materials

Restriction, ligation, and other DNA-modifying enzymes were purchased from Promega (Madison, WI) or Stratagene (LaJolla, CA). Oligo(dT) cellulose (Type III) was from Collaborative Research, Inc. (Bedford, MA), and radioactive compounds were obtained from Dupont-New England Nuclear (Boston, MA). RNAzol B was from Tel-Test, Inc. (Friendswood, TX). All other reagents were of molecular biology grade and were obtained either from Sigma (St. Louis, MO) or Amresco (Solon, OH). Nitrocellulose filters were from Sartorius (Göttingen, Germany), and x-ray film (T-Mat) was from Eastman Kodak (Rochester, NY). The *hsp*70 and *hsp*27 cDNA probes were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and labeled using a random priming kit purchased from Stratagene. The cyclophilin cRNA probe was labeled using an *in vitro* transcription kit purchased from Promega.

Animals

Young adult (4-month-old) and aged (22- to 26-month-old) male Fischer 344 rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN), housed at a constant temperature (22 C) and humidity with 12-hour light and dark cycles, and fed a standard laboratory chow (Formulab Chow, Purina Mills, St. Louis, MO). All rats were acclimated for at least 1 week before use.

Experimental Design

Experiment 1

Young adult (4-month-old) Fischer 344 rats (n = 9) were placed in individual wire-bottomed cages and fasted from food for 48 hours but allowed free access to water. Rats were killed after the 48-hour fast and 6 and 24 hours after refeeding (n = 3 rats/group). Three additional rats were killed without fasting or refeeding (controls). The stomach and ileum were removed, flash frozen in liquid nitrogen, and stored at −70 C before RNA extraction.

Experiment 2

Aged (22- to 26-month-old) Fischer 344 rats were either fed AL or given a CR diet (60% caloric intake of AL diet) for 8 weeks (n = 6 rats/group). Rats were killed, and the entire stomach and duodenum were removed, flash

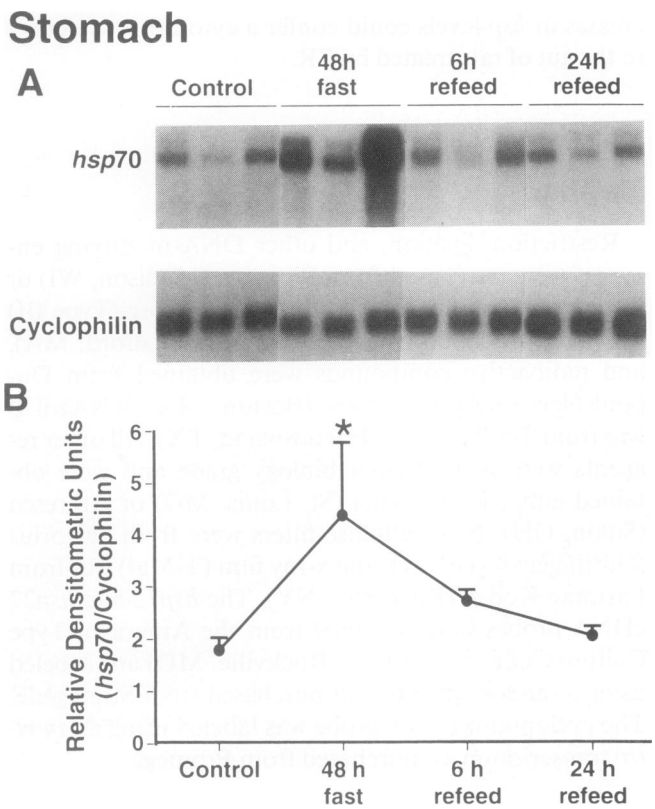

Figure 1. (A) Representative Northern blot analysis of RNA (10 μg poly(A+)/lane) extracted from the stomach of rats either fasted for 48 hours, fasted for 48 hours and then refed for 6 and 24 hours, or rats fed *ad libitum* (control). Blots were probed with cDNA probes for *hsp*70 and cyclophilin and exposed to Kodak T-mat film (Eastman Kodak, Rochester, NY) using two intensifying screens at −70 C. (B) Northern blots were quantitated using scanning densitometry and expressed as relative densitometric units after normalizing for cyclophilin expression (mean ± standard error of the mean; *$p < 0.05$ *vs.* control; n = 3 rats/group).

frozen in liquid nitrogen, and stored at −70 C before RNA extraction.

RNA Extraction and Northern Blot Analysis

Total RNA was extracted by the method of Chomczynski and Sacchi[18] using RNAzol B according to the manufacturer's conditions. Polyadenylated [poly(A)$^+$] RNA was then selected by oligo(dT) cellulose column chromatography. Northern blot analyses, using 10 μg poly(A)$^+$ RNA, were performed as described previously.[19]

The *hsp*70 and *hsp*27 complementary DNA (cDNA) probes were labeled with [α-^{32}P] deoxyadenosine triphosphate by random priming. Prehybridization, hybridization, and posthybridization procedures were performed at 43 C, as described previously.[19] To ensure integrity of the RNA samples and to control for any loading differences, Northern blots were stripped and reprobed with the constitutively expressed cyclophilin gene.[19] Hybridization signals were analyzed quantitatively using a Lynx 5000 digital image analysis system (Applied Imaging Corp., Santa Clara, CA) and normalized to cyclophilin.

Statistical Analysis

Values are expressed as the mean ± standard error of the mean and analyzed by Student's t test at the 0.05 level of significance.

RESULTS

Fasting Increases *hsp*70 mRNA Levels in Rat Stomach

To first determine whether *hsp*70 levels are altered in the rat gut by a 48-hour fasting and refeeding regimen, the entire stomach and ileum was extracted and analyzed by Northern blot. The expected 2.3-, 2.8- and 3.1-kilobase mRNA transcripts were noted in the rat stomach (Fig. 1A). The 2.3-kilobase transcript represents the constitutively expressed form of *hsp*70[20]; the 2.8- and 3.1-kilobase transcripts are the inducible forms of *hsp*70.[20] Small but detectable amounts of the inducible forms of *hsp*70 mRNA are present in chow-fed control animals and may represent a normal expression pattern in the stomach. We found by densitometric analysis that *hsp*70 mRNA levels were increased approximately threefold after a 48-hour fast, with a later return to control levels 6 and 24 hours after refeeding (Fig. 1B). In contrast to

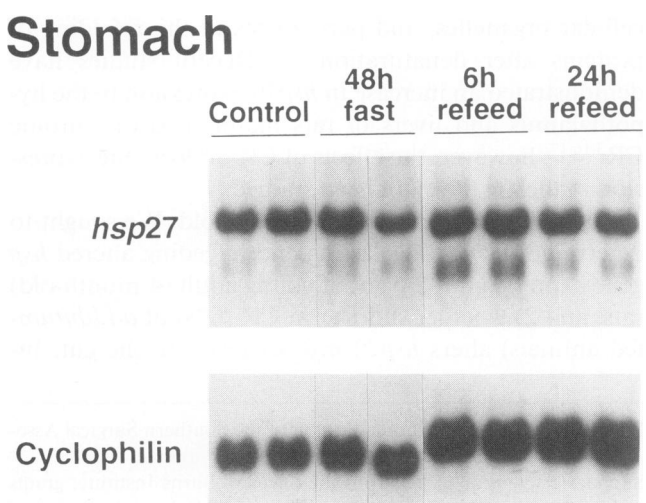

Figure 2. Northern blot analysis of RNA (10 μg poly(A)+/lane) extracted from the stomach of rats either fasted for 48 hours, fasted for 48 hours and then refed for 6 or 24 hours, or rats fed *ad libitum* (control). Blots were probed with cDNA probes for *hsp*27 and cyclophilin and exposed to Kodak T-mat film (Eastman Kodak, Rochester, NY) using two intensifying screens at −70 C.

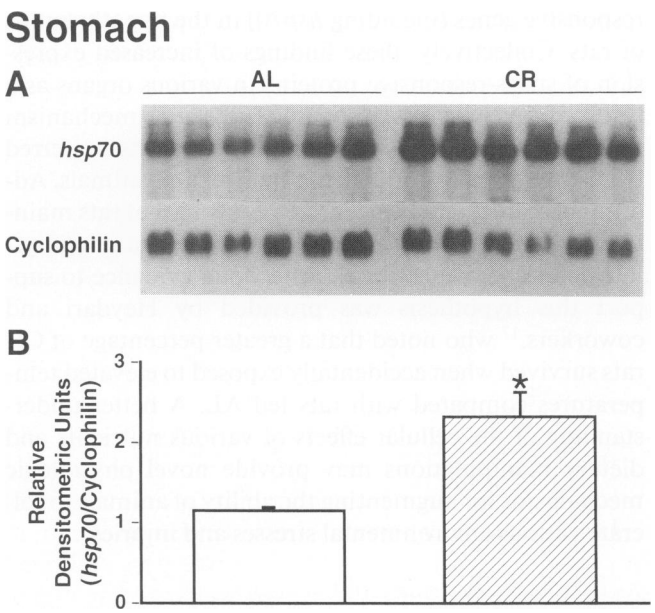

Figure 3. (A) Northern blot analysis of RNA (10 μg poly(A)+/lane) extracted from the stomach of aged rats either fed *ad libitum* (AL) or caloric restricted (CR) for 8 weeks. The blot was probed sequentially with cDNA probes for *hsp*70 and cyclophilin. (B) Northern blots for AL-fed (open bar) and CR (single-hatched bar) were quantitated using scanning densitometry and expressed as relative densitometric units after normalizing for cyclophilin (mean ± standard error of the mean; *p < 0.05 *vs.* AL fed; n = 6 rats/group).

*hsp*70, *hsp*27 levels were affected only minimally by fasting and refeeding, as shown by Northern blot analysis (Fig. 2). In addition, the increases in *hsp*70 were more pronounced in the proximal gut because only minimal changes in *hsp*70 mRNA levels were noted in the ileum after fasting (data not shown).

Taken together, our findings demonstrate a significant elevation in *hsp*70 mRNA levels with fasting; these levels quickly returned to normal after refeeding. Furthermore, the increase in mRNA levels were specific to *hsp*70 (compared with *hsp27*) and were more pronounced in the proximal gut (*i.e.*, stomach) compared with the distal small bowel.

Caloric Restriction Augments *hsp*70 mRNA Expression in the Stomach and Duodenum

Because *hsp*70 expression levels are altered dramatically by fasting, we next determined whether short-term CR (8 weeks) could alter *hsp*70 levels in the stomach and duodenum of aged rats compared with AL-fed control animals. Northern blot and subsequent densitometric analyses (Figs. 3A and 3B) demonstrated an approximate twofold increase of *hsp*70 in the stomachs of the CR rats compared with those fed AL. The RNA samples were intact and showed relatively equal loading, as demonstrated by the constitutively expressed cyclophilin gene. Similar to the stomach, *hsp*70 mRNA was increased significantly in the duodenum of the CR rats compared with AL-fed controls (Figs. 4A and 4B).

Collectively, our findings demonstrate that *hsp*70 mRNA levels are increased in the proximal gut by either an acute fast or short-term CR.

DISCUSSION

In our current study, we found that an acute 48-hour fast resulted in a marked induction of *hsp*70 mRNA levels in the stomach of adult rats; these levels rapidly returned to near-baseline levels after 6 hours of refeeding. We further found that a short-term CR regimen could elevate levels of *hsp*70 mRNA in the stomach and duodenum of aged rats. As has been postulated in other organ systems,[9-11] we speculate that the Hsps play an important cytoprotective function in the gut after an injury or stress (*e.g.*, nutrient deprivation). In addition, elevation of *hsp*70 mRNA in the gut with CR may lead to subsequent increases of Hsp protein and provide a possible cellular mechanism to explain the well-documented beneficial effects of CR.[1-3]

Cells react to physical or chemical injuries by mounting a stress response, an evolutionary mechanism de-

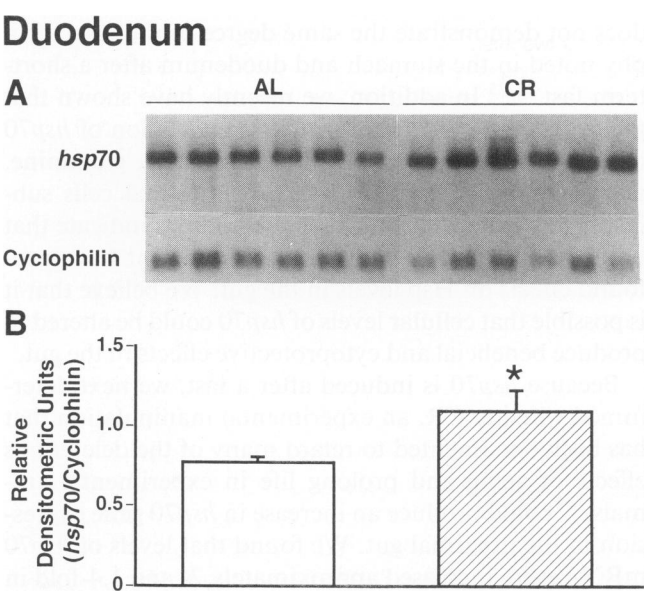

Figure 4. (A) Northern blot analysis of RNA (10 μg poly(A)+/lane) extracted from the duodenum of the same animals shown in Figure 2. The blot was probed sequentially with cDNA probes for *hsp*70 and cyclophilin. (B) Northern blots for rats fed *ad libitum* ([AL]; open bar) and caloric restricted ([CR]; single-hatched bar) were quantitated using scanning densitometry and expressed as relative densitometric units after normalizing for cyclophilin (mean ± standard error of the mean; *p < 0.05 *vs.* AL fed; n = 6 rats/group).

signed to conserve and protect vital cellular functions.[10,11] In this response, a majority of genes are expressed at lower levels or turned off completely, but a few genes (*e.g.*, the stress-responsive Hsps) are activated.[10–12,21] Morimoto and colleagues[10] have postulated that a rise in Hsp expression may be required for cells to recover from a metabolic insult. Raising the cellular levels of these molecular chaperons may facilitate the synthesis and assembly of new proteins and, more importantly, may prevent the denaturation of additional cellular proteins.[11,14,15]

In the current study, we examined the stomach, which is very susceptible to alterations in nutrients, as demonstrated by a marked mucosal atrophy that occurs after an acute (48–72 hours) fast.[22–25] We found that a 48-hour fast produced a marked induction of *hsp*70 gene expression in the stomach of rats; this effect was reversed with the readministration of food and appeared to be specific to *hsp*70 because the levels of the smaller molecular weight *hsp*27 were only minimally affected by fasting and refeeding.

Our findings of increased *hsp*70 mRNA with fasting were not entirely unexpected because other investigators have noted similar inductions in various Hsps with nutrient deprivation both *in vitro* and *in vivo*.[3,16,26] A remarkable feature, however, was that *hsp*70 levels in the distal ileum were only minimally affected by fasting. Therefore, the stomach may be more susceptible to the deleterious effects of fasting than the distal gut, which does not demonstrate the same degree of mucosal atrophy noted in the stomach and duodenum after a short-term fast.[22–25] In addition, we recently have shown that the exact type of nutrient can alter expression of *hsp*70 in gut cells.[27] For example, the amino acid, glutamine, augments *hsp* induction in IEC-6 intestinal cells subjected to a brief heat shock. These findings indicate that both the nutrient composition and amount have profound effects on Hsp levels in the gut. We believe that it is possible that cellular levels of *hsp*70 could be altered to produce beneficial and cytoprotective effects in the gut.

Because *hsp*70 is induced after a fast, we next determined whether CR, an experimental manipulation that has been documented to retard many of the deleterious effects of aging and prolong life in experimental animals,[1–3] could produce an increase in *hsp*70 gene expression in the proximal gut. We found that levels of *hsp*70 mRNA were increased approximately 2- and 1.4-fold in the stomach and duodenum, respectively, in the rats maintained on a short-term CR regimen compared with the AL-fed group. Consistent with our findings, Heydari and colleagues[17] found that CR reversed the age-related decrease in *hsp*70 induction in isolated hepatocytes from aged rats. Furthermore, Aly and coworkers[3,16] found that chronic CR resulted in the induction of various stress-responsive genes (including *hsp*70) in the hypothalamus of rats. Collectively, these findings of increased expression of stress-responsive proteins in various organs as a result of CR provides a possible molecular mechanism to explain, at least in part, the beneficial effects conferred by restricting caloric intake in experimental animals. Additional studies will be needed to confirm that rats maintained on chronic CR are indeed more resistant to physiologic or chemical stresses. Anecdotal evidence to support this hypothesis was provided by Heydari and coworkers,[17] who noted that a greater percentage of CR rats survived when accidentally exposed to elevated temperatures compared with rats fed AL. A better understanding of the cellular effects of various nutrients and dietary manipulations may provide novel physiologic mechanisms for augmenting the ability of animals to tolerate various environmental stresses and injuries.

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Discussion

DR. JOSEF E. FISCHER (Cincinnati, Ohio): Dr. Townsend, Dr. Thompson, Dr. Copeland. I would like to thank the authors for giving me the manuscript in enough time to really review it and think about it.

What you have heard this morning is Dr. Thompson, Dr. Townsend, and Dr. Evers throwing down the gauntlet in respect to this meeting and the superabundance of food. And so the argument is, is good food deleterious or not?

Caloric restriction actually does decrease a whole series of illnesses. Wes Alexander from our department continues to emphasize to me that in his studies of sepsis the animals that do best are those that only have 5% of their protein requirement. And, presumably, this absence of protein does not feed the bacteria.

They used two different models, the first of which was acute, the second chronic. And in both, heat shock protein message was increased by food deprivation, which implied to them increased cellular protection.

I will confine my remarks to the chronic group. These are old rats, 22 to 24 months. They are fat rats. And acclimatized for a week, they are likely to be hypothyroid, and I think that Is one of the critical aspects of the experimental design.

Conventional opinion implies enormous wisdom on the part of rats with respect to their food intake, much greater than the human race. But I am not sure that those qualities that are implied for rats are always there.

I have a number of questions. First, you measured message RNA with Northern blots. In our experience, message RNA expression of the amount of protein which is actually produced may be dissociated. So I would ask the authors if they did any studies as to the amount of protein which actually produced—in other words, did you do any Western blots or measure the protein by another mechanism?

Second, I am concerned as to whether or not the amount of food that they gave the chronic rats really was food deprivation. To a fat rat, 60% of the enormous amount they eat, or 60% of the *ad libitum* intake may in fact be adequate, because after a while in a cage, the rats do get hypothyroid. And, in fact, 60% may be their optimal food intake.

So my question to the authors is did they measure the weights of these animals? Did they measure any other measure of adequacy of the diet as to whether or not what they were really demonstrating was that an optimal food intake, which they may have achieved, was really what the experiment was about? Did the animals maintain the same weight, or did they lose any weight on the 60% diet?

Third, I was struck in the manuscript by the disparity between the behavior of the stomach, proximal gut, and the distal stomach, which Dr. Townsend did not emphasize in the presentation. Most of the changes that are described are in the stomach and the duodenum. Our own studies of the behavior of the gut in response to the stress of sepsis is that whereas gut protein is increased, stomach protein synthesis is decreased.

I wonder whether there is a possible relationship here with cytoprotective agents and with the disparity which is so striking in the manuscript between the behavior of the proximal gut and the distal gut.

It is an important paper. I enjoyed very much reading it and I thank the Association for the privilege of discussing it.

DR. R. DANIEL BEAUCHAMP (Nashville, Tennessee): Dr. Thompson, Dr. Copeland, Dr. Townsend. I appreciate the opportunity to review this manuscript and discuss it.

I think this is another excellent piece of work from Dr. Evers' and Dr. Townsend's laboratories. The heat shock proteins are very interesting proteins. These serve as chaperon proteins. The proteins bind other proteins to prevent them from undergoing denaturation in response to stress, such as stress of heat shock or the stress of septic shock or hypovolemic shock, and they are induced in a number of different organs. And there are approx-